IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	John T. Gray and Richard C. Mulligan	
Application No.:	09/393,795	Group Art Unit: 1636
Filed:	September 10, 1999	Examiner: G. Leffers
Confirmation No.:	3301	
For:	PACKAGING CELL LINES	

CERTIFICATE OF MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service with sufficient postage as First Class Mail in an envelope addressed to Assistant Commissioner for Patents, P.O. Box 2327, Arlington, VA 22202

on 3-11-03 Sandra Jamal
Date Signature

Sandra Jamal
Typed or printed name of person signing certificate

DECLARATION OF JOHN T. GRAY, PH.D.

UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
P.O. Box 2327
Arlington, VA 22202

Sir:

I, John T. Gray, Ph.D., of 6 Casino Road, Marblehead, Massachusetts 01945, declare and state that:

1. I am one of the inventors of the subject matter described and claimed in U.S. Provisional Application No. 60/100,022 ('022), filed September 11, 1998, U.S. Provisional

Application No. 60/100,063 ('063), filed September 12, 1998 and U.S. Application No. 09/393,795 ('795), filed September 10, 1999. The '795 application claims the benefit of the '022 and '063 applications.

2. Since the filing of the '022 application, additional experimentation relevant to this patent application has been performed by me or under my supervision. The additional work, as presented in the following section, clearly demonstrates that packaging cell lines can be produced as described in the application by co-transfecting mammalian host cells with a plasmid or retroviral nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or constitutive transport elements (CTEs).

3. The following is a description and discussion of the work carried out and of the results which demonstrates that packaging cell lines can be produced by co-transfecting mammalian host cells with a plasmid or retroviral nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

Experimental Protocol

Materials and Methods

The packaging construct pHDMHgpm2, which comprises a codon optimized HIV *gagpol*, is described in the subject application at page 13, line 3 to page 14, line 3. The codon optimized HIV *gagpol* sequence of the packaging construct pHDMHgpm2 is not operably linked to a RRE. The packaging construct pHDMHgpm2 does not contain sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The pJ6omegaPuro plasmid is described in Morgenstern, J.P. and Land, H., *Nucleic Acids Res.*, 18(4):1068 (1990). The pJ6omegaPuro plasmid encodes a selectable marker for resistance to the antibiotic puromycin (Morgenstern, J.P. and Land, H., *Nucleic Acids Res.*, 18(4):1068 (1990)). The pJ6omegaPuro plasmid does not contain DNA sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The pSV2-neo plasmid, which is available from the American Type Culture Collection (ATCC), encodes a selectable marker for resistance to the antibiotic G418 (Southern, P.J. and Berg, P., *J. Mol. Appl. Genet.*, 1:327-341 (1982)). The pSV2-neo plasmid does not contain DNA sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

293a cells (Qbiogene) are human embryonic kidney epithelial cells transformed by sheared human Ad5 DNA (Massie, B. *et al.*, *Biotechnology*, 13(6):602-608 (1995)). 293a cells contain no sequences encoding envelope proteins and no sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

293G cells are described in Ory, D.S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 93(21):11400-11406 (1996). 293G cells are human embryonic kidney epithelial cells which express the vesicular stomatitis virus glycoprotein G (VSV-G) envelope in an inducible fashion (Ory, D.S. *et al.*, *Proc. Natl. Acad. Sci. USA*, 93(21):11400-11406 (1996)). 293G cells contain DNA sequences encoding the VSV-G envelope but no sequences from lentivirus or HIV, particularly sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

Cells were transfected with the codon optimized HIV gagpol expressing packaging construct HDMHgpm2 and a plasmid expressing drug resistance (selectable marker plasmid) (pJ6omegaPuro or pSV2-neo) or with the codon optimized HIV gagpol expressing packaging construct HDMHgpm2 alone using the Effectene transfection reagent according to the manufacturer's protocol (Qiagen). Briefly, plasmid DNA (packaging construct (1 μg) and/or selectable marker plasmid (2 μl)) was mixed with Enhancer (8 μl) and EC buffer and incubated

for about 5 minutes. In the presence of 2 µl of the selectable marker plasmid, 148 µl of EC buffer was used. In the absence of the selectable marker plasmid, 150 µl of EC buffer was used. Effectene Reagent (25 µl) was then added and the mixture was incubated for 5-10 minutes to allow Effectene-DNA complexes to form. The complexes were mixed with regular media (Dulbecco's modified eagle medium (DMEM) (GIBCO/BRL) supplemented with 10% Fetal Calf Serum (FCS) (Sigma), 50 units/ml penicillin and streptomycin (GIBCO/BRL) and added directly to the cells (1×10^6). The cells were cultured for two days in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin using a humidified 37°C incubator under 5% CO_2, and then split into regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with added antibiotics to select for stable clones. Antibiotic-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones.

To analyze the production of HIV *gagpol* gene products in newly created cell lines, 2.5×10^5 cells were seeded in 6-well cluster plates and incubated for 24 hours. Supernatant was collected and assayed for p24 antigen production, a processed product of HIV *gag*. The measurement of p24 antigen quantitates the production of gag proteins by the cells. The production of gagpol proteins by the cells was measured using a standard reverse transcriptase assay (Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)).

Packaging Cell Lines Expressing Codon Optimized HIV *gagpol* Gene Products

A packaging cell line expressing codon optimized HIV *gagpol* gene products was constructed by cotransfecting 293a cells (1×10^6) with the packaging construct HDMHgpm2 (1 µg) and the pJ6omegaPuro plasmid (0.05 µg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, the cells were split into regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 1 µg/ml puromycin (Sigma) to select for stable clones. Puromycin-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Sixty-two cloned cell lines expressing *gagpol* at high levels were recovered and maintained in regular media supplemented with 1 µg/ml of

puromycin. The p24 antigen production from the best expressing clone was 106 ng/ml p24/250,000 cells/24 hour period.

A packaging cell line expressing codon optimized HIV *gagpol* gene products was also constructed by cotransfecting 293a cells (1 x 10^6) with the packaging construct HDMHgpm2 (1 μg) and the pSV2-neo plasmid (ATCC) (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, stable clones were selected in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 500 μg/ml G418 (GIBCO/BRL). G418-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Forty-eight cloned cell lines expressing *gagpol* at high levels were reccovered. The p24 antigen production from the best expressing clone was 193 ng/ml of p24/250,000 cells/24 hour period.

Packaging Cell Line Expressing Codon Optimized HIV *gagpol* Gene Products and VSV-G Envelope

A packaging cell line expressing codon optimized HIV *gagpol* gene products and the VSV-G envelope was constructed by transfecting 293G cells (1 x 10^6) with the packaging construct HDMHgpm2 (1 μg) and the pSV2-neo plasmid (ATCC) (0.05 μg) as described above. After culturing the cells for two days at 37°C and 5% CO_2, stable clones were selected in regular media (DMEM, 10% FCS, 50 units/ml penicillin and streptomycin) supplemented with 300 μg/ml G418, 1 μg/ml tetracycline (which represses expression of the VSV-G) and 2 μg/ml puromycin (which maintains the pre-existing VSV-G gene in the 293G cell line). G418-resistant clones were amplified and screened for HIV *gagpol* gene expression to select the highest expressing clones. Sixty cloned cell lines expressing *gagpol* at high levels were selected. The p24 antigen production from the best expressing clone was 138 ng/ml of p24/250,000 cells/24 hour period.

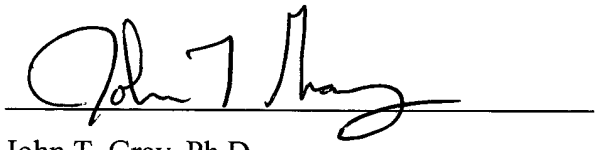
Results

The results of these experiments show that packaging cell lines can be produced as described in the application by co-transfecting mammalian host cells with a plasmid or retroviral

nucleotide sequence (packaging construct) comprising a codon optimized DNA sequence which encodes lentivirus *gagpol* proteins (e.g., HIV *gagpol* proteins) but not DNA sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or CTEs.

The establishment of these cell lines clearly demonstrates that packaging cell lines with high level stable expression of HIV *gagpol* gene products without the addition of viral sequences encoding lentivirus or HIV accessory proteins (tat, vif, vpr, vpu, nef and rev proteins and RRE) or constitutive transport elements (CTEs) can be produced without undue experimentation following the teachings of the '795 application. None of the plasmids or cell lines used in the experiments described herein contain the lentivirus or HIV accessory proteins tat, vif, vpr, vpu, nef and rev proteins and RRE. Additionally, no CTEs were included to facilitate *gagpol* gene expression. These results also demonstrate that codon optimization does in fact enable the high level expression of HIV *gagpol* in the absence of the aforementioned accessory proteins or viral sequences.

4. I declare that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true. Moreover, these statements were made with the knowledge that willful false statements and the like made by me are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.



John T. Gray, Ph.D.

March 7, 2003

Date